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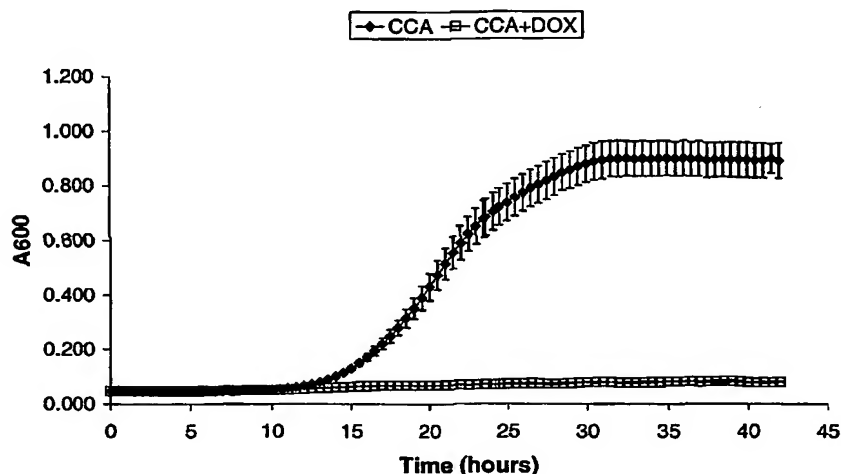
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(54) Title: CCA1 AS AN ANTIFUNGAL TARGET



(57) Abstract: The invention provides ATP(CTP) : tRNA nucleotidyltransferase (CCA1) as a novel antifungal target, screening methods for CCA1 inhibitors and their use as antifungal compounds, pharmaceutical compositions containing them and their use in medicine. Specifically in the treatment of an individual susceptible to or suffering from an anti-fungal infection. In particular the compounds find use in the treatment of topical or mucosal (e.g. thrush and vaginal candidiasis) fungal infections, e.g. caused by fungus of the Candida species, and for systemic infections, e.g. caused by fungi of Candida and Aspergillus species, such as but not limited to C. Albicans, Aspergillus flavus or Aspergillus fumigatus.

## CCA1 AS AN ANTIFUNGAL TARGET

The present invention relates to a novel antifungal target, ATP(CTP):tRNA nucleotidyltransferase (CCA1), screening methods for CCA1 inhibitors and their use as antifungal compounds, pharmaceutical compositions containing them and their use in medicine, specifically in the treatment of an individual susceptible to or suffering from an anti-fungal infection. In particular the compounds find use in the treatment of topical or mucosal (e.g. thrush and vaginal candidiasis) fungal infections, e.g. caused by fungus of the *Candida* species, and for systemic infections, e.g. caused by fungi of *Candida* and *Aspergillus* species, such as but not limited to *C. albicans*, *Aspergillus flavus* or *Aspergillus fumigatus*.

## INTRODUCTION

Fungal Pathogens

Two major fungal pathogens are those of the *Candida* species, such as but not limited to, *C. albicans*, and those of the *Aspergillus* species, such as but not limited to, *Aspergillus flavus* or *Aspergillus fumigatus*.

Fungal infections can affect humans and animals. Generally, fungal infections occur as a result of opportunistic infection of a weakened or immune-suppressed individual and these can include infections of the joints and skin. The yeast *Candida albicans* (*C. albicans*) is one of the most pervasive fungal pathogens in humans. It is the cause of an increasing financial and logistic burden on the medical care system and its providers. Although *C. albicans* is a member of the normal flora of the mucous membranes in the respiratory, gastrointestinal, and female genital tracts, it may gain dominance in such locations (e.g. upon treatment with antibacterial antibiotics, in patients with diabetes or in patients using corticosteroids) and be associated with pathologic conditions. In addition, almost all HIV-positive individuals suffer from a *Candida* infection prior to the onset of developing full-blown AIDS. The incidence of life-threatening fungal infections has increased dramatically as the population of immunocompromised individuals (including cancer, organ transplant and AIDS patients) has increased. Present therapeutic options for the treatment of these infections are limited and thus there is a need for new anti-fungal compounds with novel mechanisms of action for use in treating or preventing such fungal infections.

Antifungal drug development often relies on the screening of a large number of compounds before one or more lead compounds are found that are effective against the target fungi. Thus, it is critical for the development of these screens to define proteins essential for survival or growth of the target fungi and to discover means of purifying or producing such proteins. Thus, there is a need in the art to identify essential fungal structural or functional gene products that can serve as targets for drug intervention, and for methods for identifying useful anti-fungal agents that impair the function of these essential fungal gene products, and for compositions that can be used to treat fungal infections by preventing or inhibiting the growth of, and preferentially killing, the fungi.

Identification of "Essential" genes

Varying definitions are used in the art for what constitutes an essential gene, but the term is most frequently applied to those genes necessary for growth on rich medium. This variation in the art can be misleading and restrictive in terms of identifying gene products that constitute good antifungal targets. A significant amount of *C. albicans* genomic sequence information is available in both public (<http://www.sequence.stanford.edu/group/candida/>) and private (Incyte Genomics Inc.) databases. This can be combined with genomic sequence data from other organisms (The yeast genome directory, 1997, Nature, 387(6632 Suppl):5; Wood V, *et al*, 2002, Nature, 415(6874):871-80) and with supporting data such as the functional profiling of the *Saccharomyces cerevisiae* genome (Giaever G, *et al*, 2002, Nature, 418(6896):387-91). This bioinformatics driven approach has allowed the prediction of genes that may be

essential in *C. albicans* (Spaltmann F, *et al*, 1999, Drug Discovery Today, 4:17-26). However, even for relatively closely related organisms such as *Saccharomyces cerevisiae* and *C. albicans*, there are significant differences that make such *in silico* predictions unreliable. For example, CET1 and CDC25 are not essential in *C. albicans* despite being essential in *S. cerevisiae* (Enloe B, *et al*, 2000, J. Bacteriol., Oct, 182:20, 5730-6; Dunyak DS, *et al*, 2002, 6<sup>th</sup> ASM Conference on Candida and Candidiasis).

There are several strategies for identifying essential genes in *C. albicans* by practical methodology. Negative approaches rely on the inability to generate a strain that contains a disrupted functional target gene. The majority of genes characterised in this way rely on variations of the *URA* blaster method (Fonzi WA & Irwin MY, 1993, Genetics, 134:717-728). These techniques can be highly effective for analysing individual genes, but they may not be completely reliable. *CET1* was incorrectly reported to be essential in *C. albicans* because viable homozygous mutants could not be recovered using the *URA* blaster method (Pei, *et al*, 2001). However it has subsequently been shown not to be essential (Dunyak, *et al*, 2002). Positive approaches control the expression of the target gene either indirectly, such as using antisense RNA (De Backer MD, *et al*, 2001, Nat. Biotechnol., Mar, 19:3, 235-41), or directly such as promoter replacement with inducible promoters such as *MRP1* and *Tet* (Munro CA, *et al*, 2001, Mol. Microbiol., Mar 39:5 1414-26; Nakayama H, *et al*, 2000, Infect. Immun., Dec 68:12 6712-9).

Genome wide identification of essential genes has not been successfully applied to *C. albicans* for several reasons. These include that *C. albicans* is a diploid organism, is not capable of mating under normal circumstances, and that there are few functional transposable elements. Attempts to overcome these issues by using antisense RNA and promoter interference have had limited success (De Backer, *et al*, 2001). Therefore there is a need in the art for validated essential genes of fungal species, in particular the *Candida* species, that can be used as targets for the development of new antifungal compounds.

#### ATP(CTP):tRNA nucleotidyltransferase

The ATP(CTP):tRNA nucleotidyltransferase (CCA1) E.C.2.7.7.25 adds CCA to the 3' end of immature or damaged tRNAs and belongs to a group of tRNA processing enzymes (Martin & Hopper, 1994, Biochimie, 76(12) 1161-7). The CCA1 enzyme is encoded by the CCA1 gene and details for the fungal enzyme are provided under Accession numbers: CA1841, in the Institut Pasteur *Candida* database (<http://genolist.pasteur.fr/CandidaDB/>) which is cross-referenced with the Stanford open-reading frame (ORF) orf6.3516 (contig6-2252; <http://www.sequence.stanford.edu/group/candida/>). Synonyms for CCA1 include 4444.2, CaCCA1 and orf6.3516.

Chen J-Y, *et al*, J. Biol. Chem., 1990, 265:27, 16221-16224 describe the purification of CCA1 from *S. cerevisiae*. Wolfe CL, *et al*, J. Biol. Chem., 1996, 271:9, 4679-4686 describe mechanisms leading to alterations in the normal distribution of CCA1 in *S. cerevisiae*. Peltz SW, *et al*, Mol. and Cell Biol., 1992, 5778-5784 describe a mutation in the CCA1 gene which promotes stabilisation of mRNAs in *S. cerevisiae*.

Hanic-Joyce PJ, *et al*, Yeast, 2002, 19: 1399-1411 describes the characterisation of a gene encoding CCA1 from *Candida glabrata*.

The present invention is based on the finding that CCA1 is an essential protein for the fungal species *Candida* and *Aspergillus*. This finding demonstrates the potential for developing fungal selective CCA1 inhibitors, which can kill invading fungal organisms while sparing the host of any detrimental effects. Prior to this invention, CCA1 has not been considered as a differential target for antifungal compounds.

#### **SUMMARY OF THE INVENTION**

The present invention relates to fungal ATP(CTP):tRNA nucleotidyltransferase (hereinafter referred to as "CCA1") as a target for antifungal therapy, in particular, for antifungal therapy against

*Candida* and *Aspergillus* species. The invention also relates to a method for screening or testing for potential antifungal compounds, e.g. small molecules, by determining whether a candidate agent is capable of specifically inhibiting fungal tRNA nucleotidyltransferase function via a selective interaction with CCA1. The present invention describes the essential nature of CCA1 in *C. albicans*. It further describes the use of mechanism-based assays, with or without the use of a transformed eukaryotic organism with the CCA1 gene under the control of a heterologous promoter, to facilitate drug discovery.

Additionally, the invention relates to CCA1 inhibitor compositions and to methods for treating fungal infections, e.g. *Candida* and *Aspergillus* fungal infections, by administering to a host suffering from a fungal infection a therapeutically effective amount of a CCA1 inhibitor.

## Definitions

In the context of this invention:

"Essential gene" is defined as a fungal gene necessary for growth on rich medium.

"CCA1 inhibitor" is defined as any compound that impairs CCA1 function in the fungus. A compound that impairs CCA1 function may be one that, modulates, e.g. inhibits, the expression or activity of CCA1, interacts with CCA1 or binds to CCA1. Furthermore, a compound that modulates the expression of CCA1 may interfere with the transcription of the gene encoding CCA1 or with the translation of mRNA encoding CCA1 in target organisms. It is desirable that the compound shows specificity for fungal over host CCA1. A therapeutically effective amount of a CCA1 inhibitor is one that is sufficient to inhibit partially or fully the tRNA nucleotidyltransferase function via CCA1 of the causative fungi.

"Fragment" is defined as a fragment of a CCA1 polypeptide e.g. as provided by accession numbers CA1841 or Stanford orf6.3516, having at least 70%, more preferably it has at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% identity to the native polypeptide over the length of the fragment and which is at least ten amino acids long. An active fragment is one that retains the ability to carry out the CCA1 enzyme function.

"Function-conservative fragment" is defined as a CCA1 encoding sequence in which a given amino acid residue in the polypeptide has been changed without altering the overall conformation and function of the native polypeptide, including, but not limited to, replacement of an amino acid with one having similar physical and/or chemical properties (such as, for example, acidic, basic, hydrophobic, and the like) or polymorphisms.

"Fusion protein" unless otherwise specified, is defined as a CCA1 polypeptide, fragment or function-conservative fragment thereof fused via a covalent bond (e.g. a peptide bond), at optionally the N-terminus or the C-terminus, to an amino acid sequence of another protein (or portion thereof; preferably at least a 10, 20 or 50 amino acid portion of the protein). Preferably the polypeptide, or fragment thereof, is linked to the other protein at the N-terminus of the constant domain of the polypeptide.

"Growth" is defined as the normal growth pattern of fungi, i.e. the cell doubling time during the log phase of growth. For example, in rich media, wild-type *C. albicans* has a doubling time of approximately 60 min. Growth of the cells may be measured by following the optical density of cells in liquid media, where an increasing optical density indicates growth. Alternatively, growth can also be measured by colony formation from single cells on solid media plates.

"Viability" is defined as the ability of fungal cells to resume growth following a treatment of the cells that results in cessation of growth. One typical means by which viability is measured is by testing the ability of cells to form colonies on solid media plates.

## DETAILED DESCRIPTION OF THE INVENTION

The invention provides CCA1 as a specific target for antifungal compounds.

The methods of the invention provide a facile and specific assay to screen compounds as potential antifungal compounds, in particular, as antifungal compounds against *Candida* and *Aspergillus* species.

Thus, the invention provides a method of screening or testing for antifungal compounds, e.g. against *Candida* or *Aspergillus* species, that impair ATP(CTP):tRNA nucleotidyltransferase enzyme function (CCA1), comprising:

- a) providing fungal CCA1, preferably *Candida* or *Aspergillus* CCA1;
- b) providing one or more candidate compounds;
- c) contacting said CCA1 with said one or more candidate compounds; and
- d) determining the interaction of the candidate compound with said CCA1.

The screening method of the invention may be performed using techniques known in the art, e.g. the assay may comprise a growth inhibition assay, a binding assay or a translation inhibition assay. Binding assays include competitive binding assays, wherein the binding affinity of the candidate compound is compared with that of a known enzyme substrate for CCA1, a preferred enzyme substrate is cytosine triphosphate (CTP).

In the screening methods of the invention the candidate compound or enzyme substrate may be labelled to allow easy quantitation of the interaction between the candidate compound and the enzyme. Preferably the substrate is labelled e.g. using a radiolabel, such as but not limited to, tritium and the preferred substrate is cytosine triphosphate (CTP), as described in Example 3.

CCA1 may be cloned or purified from fungi for use in *in vitro* binding, ligand binding or translation inhibition assays. Preferably, the CCA1 is from fungal pathogens of humans and animals, such as *Candida* or *Aspergillus* species. In a particular embodiment, CCA1 may comprise a fragment, a function-conservative variant, an active fragment or a fusion protein of CCA1.

CCA1 can be purified by techniques well known to those skilled in the art. Methods for polypeptide purification include, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the fungal target protein or against peptides derived therefrom can be used as purification reagents.

CCA1 can also be provided in a transformed eukaryotic organism under the control of a heterologous promoter. Such cells can be used in growth inhibition assays. Preferably, the eukaryotic organism is *C. albicans* or *S. cerevisiae*. More preferably the organism is *C. albicans*. Briefly, a *C. albicans* strain is generated in which expression of the CCA1 gene can be tightly regulated. To do this the wild-type allele of the gene of interest is replaced with an allele that can be regulated by an exogenous agent. In general, nucleic acid manipulations and other related techniques used in practicing the present invention employ methods that are well known in the art, as disclosed in, e.g. Molecular Cloning, A Laboratory Manual (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor) and Current Protocols in Molecular Biology (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley Interscience, NY, NY, 1997).

Thus, the invention also provides a modified eukaryotic cell(s) wherein the cell(s) expresses CCA1 under the control of a heterologous promoter. In one embodiment, the CCA1 may be heterologous or homologous. Preferably, the CCA1 is homologous.

The eukaryotic cell is preferably *C. albicans* or *S. cerevisiae*, more preferably *C. albicans*.

In a specific embodiment, CCA1 may be expressed in a tetracycline-regulatable expression system (as described in Example 4). The tetracycline-regulatable expression system is an established tool for conditional expression of eukaryotic genes (Gossen MA, & H Bujard, 1992, Proc. Natl. Acad. Sci. USA, 89:5547-5551; Nagahashi S, *et al.*, 1997, Mol. Gen. Genet, 255:372-375; Nakayama H, *et al.*, 1998, Microbiology, 144:2407-2415; Nakayama H, *et al.*, 2000, Infect. Immun., Dec 68:12 6712-9). Such a system consists of two components derived from the Tn10 transposon of *Escherichia coli* (Hillen W, & A Wissmann, 1989, In Protein-nucleic acid interaction, vol. 10, Macmillan Press, London, United Kingdom, p.143-162). The first component comprises a minimal promoter element downstream of a tetracycline operator sequence (*tetO*); which replaces the natural promoter of the target gene. The second component is a transactivator, that is a fusion protein comprising a transcriptional activation domain and the tetracycline repressor protein (*TetR*).

In the absence of tetracycline, *TetR* specifically binds to *tetO* as a dimer, resulting in the activation of transcription by recruiting the transactivator to the promoter. When tetracycline is present, it binds to the TetR repressor with high affinity and inhibits dimerisation, thereby preventing binding to *tetO*. Therefore, CCA1 gene expression is activated in the absence, and repressed in the presence, of tetracycline. A synthetic tetracycline derivative, such as but not limited to, doxycycline can be used to control the expression of the CCA1 gene as described above.

The tetracycline-regulatable expression system has several advantages over alternative systems. It was derived from a prokaryotic system so it is not anticipated to show pleiotropic effects (Gossen and Bujard, 1992), it can be used in an animal host (Nakayama H, *et al.*, 1998, Microbiology, 144:2407-2415; Nakayama H, *et al.*, 2000, Infect. Immun., Dec 68:12 6712-9), it is highly specific, and non-toxic.

Such modified cells may be used in screening methods. Thus, the invention also provides a method of screening or testing for candidate anti-fungal compounds, e.g. against *Candida* or *Aspergillus* species, that impair ATP(CTP):tRNA nucleotidyltransferase enzyme (CCA1) function, comprising;

- a) providing fungal CCA1, preferably *Candida* or *Aspergillus* CCA1, in a eukaryotic cell(s) that expresses CCA1 under the control of a heterologous promoter;
- b) providing one or more candidate compounds;
- c) contacting said eukaryotic cell(s) with said one or more candidate compounds; and
- d) determining the interaction of the candidate compound with said CCA1 by assessing the effect on growth or viability of said cells.

The screening methods of the invention include both *in vitro* and *in vivo* methods. Candidate compounds which may be screened according to the methods of the invention include small molecules and peptides. The candidate compounds may be synthetic compounds, a mixture of synthetic compounds, a crude preparation, a purified preparation or an initial extract of a natural product obtained from plant, microorganism or animal sources.

The invention also provides a compound identified by the screening methods described above, which impairs CCA1 function and is referred to herein as a "CCA1 inhibitor".

CCA1 inhibitors of the invention are useful as antifungal compounds. Thus, they may be used in the treatment and prevention of various fungal infections such as topical or mucosal (e.g. thrush and vaginal candidiasis) fungal infections, caused by e.g. *Candida* species, and for systemic fungal infections, caused by e.g. *Candida* and *Aspergillus* species, such as but not limited to *C. albicans*, *Aspergillus flavus* or *Aspergillus fumigatus*.

For the purposes of this invention, the medicament can be used in the curative or prophylactic treatment of fungal infections in humans and animals, especially domestic animals such as dogs, cats, horses etc.

In addition, the CCA1 inhibitors also find use in the curative or prophylactic treatment of fungal infections in subjects who are immunosuppressed e.g. as a result of a therapy (e.g. chemotherapy or radiotherapy), organ transplant or an infection (e.g. HIV).

In additional embodiments, therefore, the present invention provides:

- 5 i) the use of a CCA1 inhibitor as an anti-fungal agent.
- ii) the use of a CCA1 inhibitor in the manufacture of a medicament for the treatment of fungal infections, such as topical or mucosal (e.g. thrush and vaginal candidiasis) fungal infections, e.g. caused by *Candida* species, and for systemic fungal infections e.g. caused by *Candida* and *Aspergillus* species, such as but not limited to, *C. albicans*, *Aspergillus flavus* or *Aspergillus fumigatus*.
- 10 iii) the use of a CCA1 inhibitor in the manufacture of a medicament for the treatment of fungal infections in a subject who is immunosuppressed, for example, as a result of a therapy (e.g. chemotherapy or radiotherapy); organ transplant or an infection (e.g. HIV).
- iv) a method for the treatment or prevention of fungal infections in a host, such as topical or mucosal (e.g. thrush and vaginal candidiasis) fungal infections, e.g. caused by *Candida* species, and for systemic
- 15 fungal infections, e.g. caused by *Candida* and *Aspergillus* species, such as but not limited to *C. albicans*, *Aspergillus flavus* or *Aspergillus fumigatus*, which comprises administering to the host a therapeutically or prophylactically effective amount of a CCA1 inhibitor.
- v) a method for the treatment or prevention of fungal infections in a subject who is immunosuppressed, for example, as a result of a therapy (e.g. chemotherapy or radiotherapy), organ
- 20 transplant or an infection (e.g. HIV) which comprises the step of administering to the subject a therapeutically or prophylactically effective amount of a CCA1 inhibitor.

In order to use CCA1 inhibitors in therapy (human or veterinary), they will normally be formulated into a pharmaceutical composition in accordance with standard pharmaceutical practice, e.g. by admixing the CCA1 inhibitor and a pharmaceutically acceptable carrier.

- 25 Thus according to a further aspect of the invention there is provided a pharmaceutical composition comprising a CCA1 inhibitor and a pharmaceutically acceptable carrier. The pharmaceutical compositions are particularly useful in the prevention or treatment of fungal infections, preferably, in the treatment of *Candida* or *Aspergillus* fungal infections.

- 30 CCA1 inhibitors may be administered to a host by any of the routes conventionally used for drug administration, for example they may be administered parenterally, orally, topically (including buccal, sublingual or transdermal) or by inhalation. The most suitable route for administration in any given case will depend on the particular CCA1 inhibitor, the infectious organism involved, the host, and the nature and severity of the disease and the physical condition of the host.

- 35 The CCA1 inhibitors may be administered in combination, e.g. simultaneously, sequentially or separately, with one or more other therapeutically active, e.g. antifungal, compounds.

- The dosage to be administered of a CCA1 inhibitor will vary according to the particular CCA1 inhibitor, the infectious organism involved, the host, the severity of the disease, physical condition of the host, and the selected route of administration; the appropriate dosage can be readily determined by a person skilled in the art. For the treatment of fungal diseases in humans and animals, the dosage may
- 40 range from 0.01 mg/kg to 750 mg/kg. For prophylactic use in human and animals, the dosage may range from 0.01 mg/kg to 100 mg/kg.

The compositions may contain from 0.1% by weight, preferably from 10-60% by weight, of the CCA1 inhibitor, depending on the method of administration.

- 45 Pharmaceutical compositions may be conveniently presented in unit dose forms containing a predetermined amount of CCA1 inhibitor per dose. Such a unit may contain for example but without limitation, 100mg/kg to 0.1mg/kg depending on the condition being treated, the route of administration and



the age, weight and condition of the host. Preferred unit dosage compositions are those containing a daily dose or sub-dose, as recited above, or an appropriate fraction thereof, of the active ingredient.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of an agent of the invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular host being treated, and that such optimums can be determined by conventional techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e. the number of doses of an agent of the invention given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determination tests.

Dosage regimens are adjusted to provide the optimum desired response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation.

Pharmaceutically acceptable carriers for use in the invention may take a wide variety of forms depending, e.g. on the route of administration.

Compositions for oral administration may be liquid or solid. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Oral liquid preparations may contain suspending agents, for example sorbitol, methyl cellulose, glucose syrup, gelatin, hydroxyethyl cellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; water; non-aqueous vehicles (which may include edible oils), for example almond oil, oily esters such as glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl *p*-hydroxybenzoate or sorbic acid; flavoring agents, preservatives, coloring agents and the like may be used.

In the case of oral solid preparations such as powders, capsules and tablets, carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like may be included. Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit form in which case solid pharmaceutical carriers are generally employed. In addition to the common dosage forms set out above, CCA1 inhibitors may also be administered by controlled release means and/or delivery devices. Tablets and capsules may comprise conventional carriers or excipients such as binding agents for example, syrup, acacia, gelatin, sorbitol, tragacanth, or polyvinylpyrrolidone; fillers, for example lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tableting lubricants, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants, for example potato starch; or acceptable wetting agents such as sodium lauryl sulphate. The tablets may be coated by standard aqueous or non-aqueous techniques according to methods well known in normal pharmaceutical practice.

Pharmaceutical compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient, as a powder or granules, or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association the active ingredient with the carrier, which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation. For example, a tablet may be prepared by compression or moulding, optionally with one or more accessory ingredients.



Compressed tablets may be prepared by compressing, in a suitable machine, the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, surface active or dispersing agent. Moulded tablets may be made by moulding, in a suitable machine, a mixture of the powdered compound moistened with an inert liquid diluent. Desirably, each tablet contains from about 1 mg to about 500 mg of the active ingredient and each cachet or capsule contains from about 1 to about 500 mg of the active ingredient.

Compositions comprising a CCA1 inhibitor may also be prepared in powder or liquid concentrate form. Conventional water soluble excipients, such as lactose or sucrose, may be incorporated in the powders to improve their physical properties. Thus, particularly suitable powders of this invention comprise 50 to 100% w/w, and preferably 60 to 80% w/w of the combination and 0 to 50% w/w and preferably 20 to 40% w/w of conventional excipients. When used in a veterinary setting such powders may be added to animal feedstuffs, for example by way of an intermediate premix, or diluted in animal drinking water.

Liquid concentrates of this invention for oral administration suitably contain a water-soluble compound combination and may optionally include a pharmaceutically acceptable water miscible solvent, for example polyethylene glycol, propylene glycol, glycerol, glycerol formal or such a solvent mixed with up to 30% v/v of ethanol.

Pharmaceutical compositions suitable for parenteral administration may be prepared as solutions or suspensions of the CCA1 inhibitors in water suitably mixed with a surfactant such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include aqueous or non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the composition isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. Extemporaneous injection solutions, dispersions and suspensions may be prepared from sterile powders, granules and tablets.

The compositions may be presented in unit-dose or multi-dose containers, for example in sealed ampoules and vials and to enhance stability, may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. The sterile liquid carrier may be supplied in a separate vial or ampoule and can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g. glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils. Advantageously, agents such as a local anaesthetic, preservative and buffering agents can be included the sterile liquid carrier.

Pharmaceutical compositions adapted for topical administration may be formulated as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, impregnated dressings, sprays, aerosols or oils, transdermal devices, dusting powders, and the like. These compositions may be prepared via conventional methods containing the active ingredient. Thus, they may also comprise compatible conventional carriers and additives, such as preservatives, solvents to assist drug penetration, emollients in creams or ointments and ethanol or oleyl alcohol for lotions. Such carriers may be present as from about 1% up to about 98% of the composition. More usually they will form up to about 80% of the composition. As an illustration only, a cream or ointment is prepared by mixing sufficient quantities of hydrophilic material and water, containing from about 5-10% by weight of the compound, in sufficient quantities to produce a cream or ointment having the desired consistency.

Pharmaceutical compositions adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis.

For applications to external tissues, for example the mouth and skin, the compositions are preferably applied as a topical ointment or cream. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water cream base or a water-in-oil base.

Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouth washes.

Pharmaceutical compositions adapted for topical administration to the eye include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent. They also include topical ointments or creams as above.

Pharmaceutical compositions suitable for rectal administration wherein the carrier is a solid are most preferably presented as unit dose suppositories. Suitable carriers include cocoa butter or other glyceride or materials commonly used in the art, and the suppositories may be conveniently formed by admixture of the combination with the softened or melted carrier(s) followed by chilling and shaping moulds. They may also be administered as enemas.

Pharmaceutical compositions adapted for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray compositions. These may comprise emollients or bases as commonly used in the art.

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

The following examples are to be construed as merely illustrative and not a limitation on the scope of the invention in any way. The specification refers to the figure in which:

**Figure 1:** This shows the solid phase growth of a recombinant *C.albicans* strain wherein the CCA1 gene is under the regulation of a tetracycline-repressible promoter. The images represent two days growth at 30°C on SC-U plates with, either no supplement (-DOX), or supplemented with the tetracycline analogue doxycycline 20µg/ml (+DOX). The effect of strong induction or tight repression of CCA1 gene expression on colony formation is observed in the absence and presence of doxycycline, respectively.

**Figure 2:** This shows the liquid phase growth of a recombinant *C.albicans* strain wherein the CCA1 gene is under the regulation of a tetracycline-repressible promoter. Growth is in SC-U medium at 25°C with, either no supplement (-DOX), or supplemented with the tetracycline analogue doxycycline, 20µg/ml (+DOX). The effect of strong induction or tight repression of CCA1 gene expression on growth is observed in the absence and presence of doxycycline, respectively.

## EXAMPLES

### Example 1: Expression of CCA1

The CCA1 ORF was cloned into pGex-6P-1 (Pharmacia Biotech) to enable expression as a 5' GST fusion protein. Host *E. coli* used were BLR(DE3)pLysS (Novagen). *E. coli* harbouring the expression plasmid were grown at 30°C to mid log phase (OD600 = 0.6) and induced with IPTG (0.3mM) for approximately 4 h.

### Example 2: Purification of CCA1

*E. coli* BLR cells from 10L of culture were harvested by centrifugation at 6000 x g for 10min. The cell pellets were frozen at -80°C, thawed and resuspended in 150ml of buffer A (20mM Hepes (pH7.4), 5mM DTT, 140mM NaCl, 1mM EDTA, 10% (v/v) glycerol, 0.02% (w/v) sodium azide, and a

protease inhibitor cocktail consisting of 1mM benzamidine, 1mg.ml<sup>-1</sup> each of pepstatin, antipain and leupeptin, 0.2mM PMSF, Complete (Roche) and general protease inhibitor cocktail (Sigma).

The extract was sonicated in 3x10s bursts to reduce viscosity. Triton X-100 was added to 1% followed by centrifugation at 100 000 x g for 15 min. The supernatant was diluted to 400 ml with buffer A and was applied to a 5ml glutathione Sepharose column linked to an AKTA FPLC system (Amersham Biosciences). The column was extensively washed with buffer B (20mM Hepes (pH 7.4), 1mM DTT, 1mM EDTA, 10% glycerol, 0.02% sodium azide) containing 0.5M NaCl, then with buffer B. GST-CCA1 was eluted with buffer C (20mM Hepes, (pH 8), 1mM DTT, 1mM EDTA, 10% glycerol, 0.02% sodium azide and 20mM reduced glutathione) and collected in 1.5ml fractions.

Fractions containing GST-CCA1 were pooled and incubated with PreScission protease (2.5U/mg GST-CCA1) (Amersham Biosciences) overnight at 4°C. The protein was then loaded onto a 1ml column of Q-Sepharose and eluted with a linear gradient of 0-500mM NaCl in buffer B. Fractions containing CCA1 were pooled and concentrated using a 50K cut-off centrifugal filtration device. The protein was then re-applied to 0.5ml of glutathione sepharose to remove residual GST and GST-CCA1.

### **Example 3: CCA1 assay**

#### **3.1 Preparation of the tRNA substrate**

The tRNA substrate was prepared according to the method of Zubay and Takanami (1964) (Zubay G, & Takanami M, 1964, Biochem. Biophys. Res. Commun., Mar 26, 15:3, 207-13). The 3'-ends of the tRNA were trimmed to allow the addition of both CTP and ATP by CCA1. In brief, 100mg tRNA (Sigma, grade XXI) was incubated for 1h at 20°C in 25ml 100mM Tris-HCl pH 9.0 containing 1.2 units snake venom phosphodiesterase I. The reaction was stopped by means of a phenol:chloroform extraction and rRNA fragments and degradation products removed by binding tRNA to Q-Sepharose in 20mM Tris-HCl pH 7.5 (2mg of tRNA/ml column; Amersham Pharmacia Biotech). The column was washed with 0.4M NaCl, 20mM Tris-HCl pH 7.5 and the tRNA eluted with a 0.4-1.0M NaCl gradient. Following ethanol precipitation, the pooled tRNA fractions were resuspended in water and adjusted to 1mg/ml final concentration.

#### **3.2 Assay conditions**

The ATP(CTP):tRNA nucleotidyltransferase assay was based on the method of Chen JY, *et al*, (1990, J. Biol. Chem., Sep 25, 265:27, 16221-4). The reaction was started by adding 1µg of the CCA1 enzyme to the reaction mixture (60µl final volume in 96 well plates) containing 50mM Glycine/NaOH (pH9.4), 10mM MgCl<sub>2</sub>, 2µg of tRNA substrate, 0.8mM <sup>3</sup>H CTP (1.25 µCi), 1% DMSO, and candidate compounds as required. Following incubation at 37°C for 10 min, the reaction was stopped by adding 60µl ice cold 2M HCl. Then 100µl from each well was transferred to 96 well GF/B plates (Packard Unifilter 96). Using a vacuum manifold, the reaction mixture was sucked through. The wells were then washed twice with 100µl 2M HCl followed by twice with 100µl ice cold ethanol. When the plate was dry the underside was sealed and 50µl of Microscint (Packard) scintillation fluid added to each well. The incorporated tritium was then measured using a Packard TopCount.

### **Example 4: CCA1 as an essential gene product**

#### **4.1 Construction of the tetracycline-regulatable expression system**

*C. albicans* CAI8 was used as the parental strain for all manipulations. The parental strain was constructed to constitutively express a codon-optimised tetracycline transactivator, consisting of TetR fused to the viral VP16 transcriptional activation domain (Gari E, *et al*, 1997, Yeast, 13:837-848), from the chromosomal enolase promoter (Mason AB, *et al*, 1993, J. Bacteriol., 175: 2632-2639). One copy of the target gene, *CCA1*, was disrupted in the transactivator expressing strain using the standard *URA*-blaster method (Fonzi WA & Irwin MY, 1993, Genetics, 134:717-728). The promoter region of the other *CCA1* allele was then replaced with the minimal promoter element containing the tetracycline operator

sequence *tetO*. Both *in vivo* and *in vitro* this system enables strong induction and tight repression of *CCA1* gene expression in the absence and presence, respectively, of the tetracycline analogue doxycycline.

#### 4.2 *In vitro* validation experiments

The essential nature of *CCA1* was determined by assessing the growth and viability of the *C. albicans* strain modified to include a tetracycline-regulatable *CCA1*-expression system as described above (section 4.1). A single fresh colony (grown at 30°C in rich medium in the absence of tetracycline) was used to streak fresh plates containing synthetic complete medium minus uracil (SC-U) (Qbiogene), plus 2% agar, plus or minus 20 µg/ml doxycycline as indicated. Growth was scored after 2 days at 30°C (Figure 1). No colonies were observed under the conditions where *CCA1* expression was repressed (i.e. in the presence of doxycycline).

Growth of the tetracycline-regulatable *CCA1*-expression system *C. albicans* strain in liquid SC-U medium was also assessed. The inoculum was a 1:100 dilution of an overnight culture adjusted with PBS to an optical density at 600nm =1 and stored at 4°C. Growth, at 25°C, plus or minus 20 µg/ml doxycycline, was measured at 30 min intervals over a 43h time period or until the growth had noticeably reached a plateau. Growth curves were recorded in 96 well plates in a Wallac plate reader (600nm, 25°C heated stage, 2mm orbital shaking pattern) (Figure 2). Again, no significant growth was observed under the conditions where *CCA1* expression was repressed (i.e. in the presence of doxycycline).

#### Example 5: *CCA1* as an essential gene product: *In vivo* validation experiment - Murine model of systemic infection with *C. albicans* conditional mutants

Several reproducible animal models have been described including those of rat vaginal and oral Candidiasis (Calderone RA & Braun PC, 1991, Microbiol. Rev., 55:1-20). However, the most commonly used model is the murine model of hematogenously inoculated, disseminated Candidiasis (Ghannoum MA, *et al*, 1995, Infect. Immun., 63:4528-4530). In the murine disseminated model, a single dose of organism is inoculated via the tail vein. The end points for this model are survival of animals and tissue counts of *C. albicans* (generally the kidneys).

To further validate the essential nature of the *CCA1* gene, the conditional mutants of *C. albicans* strains (as described in Example 4, section 4.1), wherein the *CCA1* gene is under the control of a tetracycline repressible promoter, may be tested to determine whether they are attenuated in an immuno-competent murine model of infection (Ghannoum *et al*, 1995) as follows:

Initially the organisms are grown in the absence of DOX, since under these conditions they would express the *CCA1* gene. These organisms are then used to inoculate two groups of 12 of mice. One group is treated with DOX, in which expression of the *CCA1* gene is repressed, and the second group of mice is treated with water (control) wherein the gene continued to be expressed. The mice used are single sex BALB/c mice, Harlan, 4 weeks old and weighing between 19-22 g.

Infective doses of *C. albicans* are injected into the tail vein. The inocula are from saline-washed fresh stationary phase cultures grown in NGY medium [0.1% neopeptone, 0.4% glucose, 0.1% yeast extract] for 18-24 h at 30°C. Yeasts grown in this way have a viable count of  $2 \times 10^7$  CFU/ml  $\pm 0.3 \times 10^7$  CFU/ml and can easily be adjusted to the desired concentration with saline. The concentration is checked by spectrophotometry and verified by viable counts. The volume injected is the same across the doses. An infective dose of  $1 \times 10^6$  CFU/mouse is used. This infective dose has previously been shown to give a mean survival time of 5-7 days in BALB/c mice.

Animals are fed food and water *ad libitum* throughout the course of experiment. In the DOX-treated group (+DOX), mice are administered with DOX (2 mg/ml) dissolved in 5% sucrose solution as drinking water from 2 days before the inoculation of *C. albicans* cells. The mice are known to drink approximately 5 ml of sucrose solution every day. Under this regimen, the concentrations of DOX in

serum, liver, and kidney are maintained at more than 2 mg/ml of serum, 8 mg/g of liver, and 10 mg/g of kidney, respectively (Nakayama H, *et al*, 1998, Microbiology, 144:2407–2415.) Percent survival is followed over 28 days with daily body weight monitoring. Differences between the effects of *C. albicans* with the CCA1 gene active (-DOX) or repressed (+DOX) *in vivo* are monitored by mouse survival, kidney burdens of viable fungi, and changes in body weight relative to baseline.

5

## CLAIMS

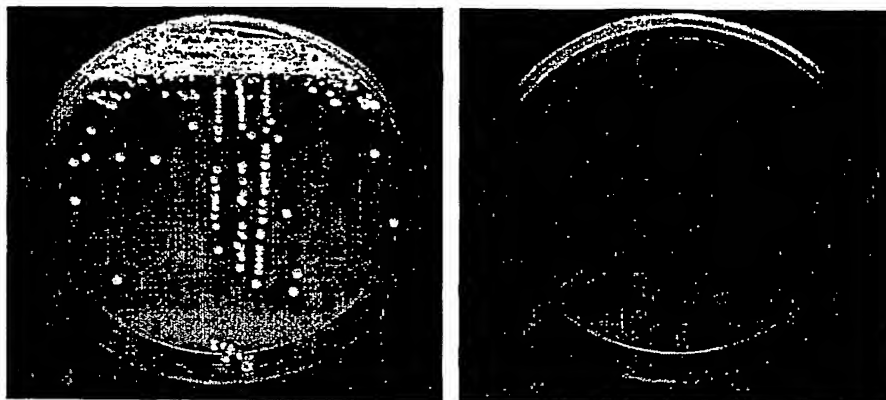
1. A method of screening or testing for candidate anti-fungal compounds that impair ATP(CTP):tRNA nucleotidyltransferase enzyme (CCA1) function, comprising:
  - 5 a) providing fungal CCA1;
  - b) providing one or more candidate compounds;
  - c) contacting said CCA1 with said one or more candidate compounds; and
  - d) determining the interaction of the candidate compound with said CCA1.
- 10 2. A method according to claim 1 wherein the CCA1 comprises a fragment, a function-conservative variant, an active fragment or a fusion protein of CCA1.
3. A method according to any one of claims 1 or 2, wherein the fungal CCA1 is from fungus of *Candida* or *Aspergillus* species.
- 15 4. A modified eukaryotic cell(s) wherein the cell(s) expresses fungal CCA1 under the control of a heterologous promoter.
5. The cell according to claim 4 which is a *C. albicans* cell.
- 20 6. The cell according to any one of claims 4 or 5, wherein the CCA1 is homologous.
7. The cell according to any one of claims 4 to 5, wherein the CCA1 comprises a fragment, a function-conservative variant, an active fragment or a fusion protein of CCA1.
- 25 8. A method of screening or testing for candidate anti-fungal compounds that impair ATP(CTP):tRNA nucleotidyltransferase enzyme (CCA1) function, comprising:
  - a) providing fungal CCA1 in a eukaryotic cell(s) as defined in any one of claims 4 to 7;
  - b) providing one or more candidate compounds;
  - 30 c) contacting said eukaryotic cell(s) with said one or more candidate compounds; and
  - d) determining the interaction of the candidate compound with said CCA1 by assessing the effect on growth or viability of said cells.
9. A compound identified by the method of claims 1, 2, 3 or 8, which impairs CCA1 function for use as an antifungal compound.
- 35 10. A pharmaceutical composition comprising a CCA1 inhibitor and a pharmaceutically acceptable carrier.
- 40 11. *Candida* or *Aspergillus* CCA1 as a specific target for antifungal compounds.
12. The use of a CCA1 inhibitor, in the manufacture of a medicament for the treatment of fungal infections.
- 45 13. The use of a CCA1 inhibitor, in the manufacture of a medicament for the treatment of fungal infections in a subject who is immunosuppressed.

14. The use according to claim 12 or 13 wherein the fungal infection is a topical, mucosal or systemic fungal infection.
- 5 15. - The use according to claim 14 wherein the topical or mucosal fungal infection is caused by species of *Candida* or the systemic fungal infection is caused by species of *Candida* or *Aspergillus*.
16. The use according to any one of claims 12 to 15 wherein said compound impairs fungal CCA1 function to a greater extent than host CCA1 function.



Figure 1

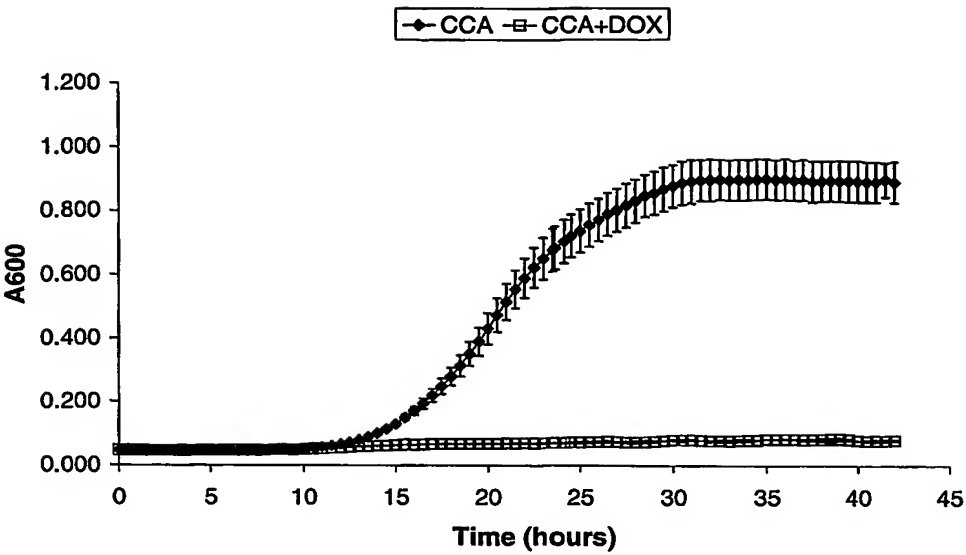
*CCA1*



- DOX

+ DOX

Figure 2



# INTERNATIONAL SEARCH REPORT

Inter. Application No  
PCT/GB 03/05373

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 G01N33/50 C12Q1/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, WPI Data, PAJ, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02 02784 A (ANADYS PHARMACEUTICALS INC) 10 January 2002 (2002-01-10) abstract claims 11,34 page 2, line 10 -page 7, line 16 page 34, line 1 -page 37, line 6 ---	1-8
X	HANIC-JOYCE PAMELA J ET AL: "Characterization of a gene encoding tRNA nucleotidyltransferase from Candida glabrata." YEAST, vol. 19, no. 16, December 2002 (2002-12), pages 1399-1411, XP002276436 ISSN: 0749-503X (ISSN print) abstract --- -/--	11

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*G\* document member of the same patent family

Date of the actual completion of the international search

8 April 2004

Date of mailing of the international search report

26/04/2004

Name and mailing address of the ISA

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## INTERNATIONAL SEARCH REPORT

Intel. Application No

PCT/GB 03/05373

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE BACKER MARIANNE D ET AL: "Recent developments in molecular genetics of <i>Candida albicans</i> " ANNUAL REVIEW OF MICROBIOLOGY, 2000, pages 463-498, XP002276431 Annual Reviews {a}, 4139 El Camino Way, Palo Alto, CA, 94303-0139, USA Series: Annual Review of Microbiology (ISSN 0066-4227) ISBN: 0-8243-1154-1 (cloth) abstract	1-8
A	DE BACKER M D ET AL: "Genomic profiling of the response of <i>Candida albicans</i> to itraconazole treatment using a DNA microarray." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. UNITED STATES JUN 2001, vol. 45, no. 6, June 2001 (2001-06), pages 1660-1670, XP002215551 ISSN: 0066-4804 abstract	1-8
A	DE BACKER MARIANNE D ET AL: "An antisense-based functional genomics approach for identification of genes critical for growth of <i>Candida albicans</i> " NATURE BIOTECHNOLOGY, vol. 19, no. 3, March 2001 (2001-03), pages 235-241, XP002276432 ISSN: 1087-0156 abstract	1-8
A	DE BACKER MARIANNE D ET AL: "RNA-mediated gene silencing in non-pathogenic and pathogenic fungi" CURRENT OPINION IN MICROBIOLOGY, vol. 5, no. 3, June 2002 (2002-06), pages 323-329, XP002276430 ISSN: 1369-5274 abstract	1-8
A	WO 01 60975 A (BOONE CHARLES ;BUSSEY HOWARD (CA); JIANG BO (CA); ROEMER TERRY (CA) 23 August 2001 (2001-08-23) abstract	1-8

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 9,10,12-16

Present claims 9,10, 12-16 relate to a compound defined by reference to a desirable characteristic or property, namely "identifiable by the method of claims 1-3 or 8".

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for no such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the methods of claims 1-3 and 8.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 03/05373

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 9, 10, 12-16  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Inter  
nal Application No  
PCT/GB 03/05373

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0202784	A	10-01-2002	US 6531289 B1	11-03-2003
			CA 2408829 A1	10-01-2002
			EP 1292689 A1	19-03-2003
			WO 0202784 A1	10-01-2002
WO 0160975	A	23-08-2001	AU 4320401 A	27-08-2001
			CA 2398861 A1	23-08-2001
			EP 1292668 A2	19-03-2003
			JP 2003523197 T	05-08-2003
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